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# PKA Enhances the Acute Insulin Response Leading to the Restoration of Glucose Control

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**Diabetes arises from insufficient insulin secretion and failure of the β-cell mass to persist and expand. These deficits can be treated with ligands to Gs-coupled G-protein-coupled receptors that raise β-cell cAMP. Here we studied the therapeutic potential of β-cell cAMP-dependent protein kinase (PKA) activity in restoring glucose control using β-caPKA mice. PKA activity enhanced the acute insulin response (AIR) to glucose, which is a primary determinant of the efficacy of glucose clearance. Enhanced AIR improved peripheral insulin action, leading to more rapid muscle glucose uptake. In the setting of pre-established glucose intolerance caused by diet-induced insulin resistance or streptozotocin-mediated β-cell mass depletion, PKA activation enhanced β-cell secretory function to restore glucose control, primarily through augmentation of the AIR. Enhanced AIR and improved glucose control were maintained through 16 weeks of a high-fat diet and aging to 1 year. Importantly, improved glucose tolerance did not increase the risk for hypoglycemia, nor did it rely upon hyperinsulinemia or β-cell hyperplasia, although PKA activity was protective for β-cell mass. These data highlight that improving β-cell function through the activation of PKA has a large and underappreciated capacity to restore glucose control with minimal risk for adverse side effects.**

ISLET STUDIES

Insulin release from β-cells of the pancreatic islets of Langerhans is fundamental to maintain glucose control, and diabetes develops when insulin secretion is insufficient (1–3). Diminished peripheral insulin sensitivity requires that β-cells compensate with increased insulin release to maintain glucose control (3,4). This compensation requires either a functional increase in the capacity of individual β-cells to secrete insulin or an increase in total β-cell number. However, in type 2 diabetes mellitus (T2DM), there is a failure of these compensatory mechanisms. Current diabetes therapies delay the progression of the disease but do not reverse or even arrest the course of the diabetes, most likely due to continued decline at the β-cells (5,6). T2DM therapies are needed to augment insulin release (β-cell function) and/or to target β-cell mass for protection and expansion (7).

Insulin secretion in response to glucose is biphasic, with an acute burst of release lasting up to 10 min followed by a sustained release that is maintained while glucose remains elevated (8–10). The magnitude of the acute phase has been shown to be the primary determinant of the rate of glucose clearance (11). Acute phase insulin release is diminished in individuals with T2DM and, importantly, is also decreased in individuals with impaired fasting glucose and in relatives of individuals with T2DM, indicating that it is an early manifestation of β-cell dysfunction (11–15). Both phases of insulin release are augmented by a rise in cAMP induced by agents such as incretins, which are gut-derived peptide hormones (GLP-1 and glucose-dependent insulinotropic peptide) (16–18). Incretins contribute up to 70% of insulin release in response to orally ingested glucose (4), and their administration to individuals with impaired glucose tolerance can restore phasic insulin release (19). Incretins mediate their actions via their cognate Gαs-coupled G-protein-coupled receptors (GPCRs) (20), which raise cAMP

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and activate two signaling systems: the cAMP-dependent protein kinase (PKA) and the exchange proteins activated by cAMP. Signaling via both PKA and the exchange proteins activated by cAMP have been reported to potentiate insulin secretion and protect β-cell mass, although recent data suggest that PKA is the major cAMP-dependent regulator of β-cell function (16,21,22). The beneficial effects of incretins upon insulin secretion and β-cell mass and the clinical success of incretin-based therapies has spurred interest in the potential of other GPCRs to provide T2DM therapies (23,24). However, the complexity of GPCR signaling is highlighted by studies of the GLP-1 receptor, an important target of T2DM therapies. The GLP-1 receptor is generally considered to act via cAMP downstream of its Gs-coupled receptor; however, it can also regulate insulin secretion and β-cell mass expansion via β-arrestin independently of cAMP (25,26) and, alternatively, can couple to Gi and Gq (27).

Raising β-cell cAMP levels is a goal of diabetes therapies that is both already in clinical use and at various stages of development. Although targeting specific outcomes downstream of GPCRs requires a close dissection of the key GPCR signaling pathways, the therapeutic potential of specifically activating PKA, the major mediator of the cAMP signaling in β-cells, has not been established. We previously generated a mouse model (β-caPKA mice) to study the PKA pathway in β-cells (16). Our initial characterization of this model showed that β-cell-specific induction of PKA activity enhanced β-cell secretory function, with a prominent effect upon the acute insulin response (AIR). This mouse model allows PKA induction at physiological levels to study β-cell-specific effects of PKA activation. Here we used these mice to therapeutically target glucose control impaired in mice through diet-induced insulin resistance, aging, and β-cell mass depletion. Our data show that PKA-mediated enhancement of β-cell secretory function was sufficient to retain and restore glucose control, primarily through enhancement of the AIR. Importantly, these effects were independent of β-cell mass hyperplasia and avoided hyperinsulinemia, hypoglycemia, and β-cell exhaustion. This study shows that developing therapies to specifically enhance the profile of insulin secretion can provide significant benefit to prevent and reverse impaired glucose tolerance. The activation of PKA provides a mechanism by which these goals can be achieved.

## RESEARCH DESIGN AND METHODS

### Animals

β-caPKA mice were generated by crossing heterozygous PKA-CαR mice (28) with heterozygous MIP-CreERT mice (29), resulting in four offspring genotypes: wild type, PKA-CαR, MIP-CreERT, and β-caPKA, which carry both the PKA-CαR allele and the MIP-CreERT transgene. Wild-type, MIP-CreERT, and PKA-CαR offspring littermates were used to control these studies. PKA-CαR and MIP-CreERT parental strains were maintained as heterozygotes by back breeding to C57Bl/6J mice carrying the Rosa/LacZ allele (B6.129S4-Gt(ROSA)26Sor$^{Tm1Sor}$/J; The Jackson Laboratory stock 3474). Mice were maintained in the University of Chicago animal facility under the day-to-day care of facility staff and according to a University of Chicago Institutional Animal Care and Use Committee–approved protocol. Standard chow diet was 24% protein, 18% fat, and 58% carbohydrate, by calorie (Harlan Teklad catalog number 2918). Mice fed a high-fat diet (HFD) received 20% of calories from protein, 45% of calories from fat, and 35% of calories from carbohydrate (Research Diets Inc. catalog number D12451). The PKA-CαR allele was induced by administering tamoxifen at 10 weeks of age, except where specified, by injecting 3 mg i.p. tamoxifen freshly dissolved in corn oil at 20 mg/mL on 3 alternate days. Freshly prepared streptozotocin (STZ) at 10 mg/mL in 50 mmol/L citrate buffer (pH 5.2) was administered by intraperitoneal injection at 90 mg/kg body weight, with untreated control mice receiving citrate buffer only. Intraperitoneal glucose tolerance tests (IPGTTs) were performed on 16-h-fasted mice by injecting 25% D-glucose at 1 g/kg of body weight, except where specified. Blood for glucose and insulin measurements was collected from the tail vein.

### Endogenous Insulin Signaling

Insulin action in skeletal muscle was determined by giving 24-h-fasted mice (4–6 months old, standard chow diet) a 3 g/kg i.p. glucose challenge, sacrificing mice at the indicated times, harvesting quadriceps, and freezing in liquid nitrogen. Ground tissue was sonicated in 240 mmol/L Tris-acetate, 1.0% SDS, 0.5% glycerol, 5 mmol/L dithiothreitol, protease inhibitors (Sigma-Aldrich catalog number P8340), phosphatase inhibitor (Santa Cruz catalog number sc-45044), 1 mmol/L $Na_3VO_4$, 10 mmol/L β-glycerophosphate; boiled for 10 min; and sonicated again. Lysates were resolved by gel electrophoresis and transferred to nitrocellulose. These membranes were probed for Akt phosphorylated at threonine 308 and serine 473 (Cell Signaling catalog numbers 4056 and 4060, respectively), total Akt (Cell Signaling catalog number 9272), and tubulin (Cell Signaling catalog number 2128).

### Glucose Uptake

Glucose uptake into muscle was measured in β-caPKA and littermate controls 15 min after a 2-deoxy-D-[1-$^{14}$C]glucose–labeled 2 g/kg D-glucose challenge. These analyses were performed at the Mouse Metabolic Phenotyping Center within the Yale University School of Medicine under standard conditions for that center.

### Measurement of β-Cell Area, Pancreatic Insulin Content, and β-Cell Proliferation

Pancreata fixed for 4 h in 4% paraformaldehyde were embedded in paraffin, and 5-μm sections stained for insulin (Cell Signaling catalog number 3014). Images were collected on a Cri Pannoramic whole slide scanner and analyzed using Pannoramic Viewer 1.15 software. A minimum of 3 mice and 15 slides (30 sections) were analyzed per group, with slides collected at 50 μm intervals. Total pancreatic insulin content was measured by ELISA (ALPCO) in extracts of the entire pancreata sonicated in 0.1 mol/L HCl. β-Cell proliferation was measured in pancreatic sections fixed and

sectioned as above, using sequential staining for Ki67 (Leica catalog number KI67P-CE; 1:250) then insulin (Cell Signaling catalog number 3014; 1:1,000).

### Statistical Analysis

Data, expressed as mean ± SD, were analyzed by Student two-sample unpaired *t* tests, one-way ANOVA, and two-way ANOVA with Bonferroni post hoc tests (GraphPad Software). $P < 0.05$ was considered significant.

## RESULTS

### PKA Activity Potentiates the AIR

β-caPKA mice have β-cell specific, Cre-inducible increased PKA activity upon tamoxifen administration (16). PKA activity is increased through the expression of an activated PKA α catalytic subunit (PKA-Cα) that was generated by a targeted integration of the endogenous PKA-Cα allele, which leaves PKA-CαR expression under the control of the endogenous *cis*-acting elements (28). β-Cell specific, tamoxifen-inducible Cre expression was obtained using MIP-CreERT mice (29). β-caPKA mice and littermate controls were raised to 10 weeks of age before receiving tamoxifen to induce increased PKA activity. We have previously shown that induction of the PKA-CαR allele increases basal PKA activity 10-fold, equivalent to ~6% of maximal islet PKA activity (16). In response to an IPGTT, 13-week-old β-caPKA mice exhibited potentiation of the acute phase of insulin secretion, most notably at 2 min (Fig. 1*A*). This led to improved glucose tolerance (Fig. 1*B*) and lowered exposure to glucose (Fig. 1*C*), as we have reported previously (16). β-caPKA mice placed on an HFD for 16 weeks, or fed a standard chow diet until the age of 52 weeks, retained the enhanced insulin release at 2 min (Fig. 2*A* and *B*). Glucose tolerance was similarly sustained (Fig. 2*C* and *D*). Ad libitum-fed glucose levels in β-caPKA mice were lower than littermates in both mice fed an HFD (Fig. 2*E*) (130 ± 18 vs. 164 ± 32 mg/mL; $n$ = 12 mice/group) and mice aged to 52 weeks (Fig. 2*G*) (120 ± 18 vs. 147 ± 25 mg/mL; $n \geq 14$ mice/group). Circulating insulin did not differ between β-caPKA mice and controls maintained for 16 weeks on an HFD (Fig. 2*F*) (2,697 ± 2,717 vs. 3,072 ± 1,644 pg/mL; $n$ = 12 mice/group). In mice aged to 52 weeks, basal PKA activity remained enhanced (Fig. 2*H*) (β-caPKA 97 ± 12 vs. controls 15 ± 12 pmol phosphate/min/mg protein; $n$ = 6 mice/group; $P < 0.0001$) to a degree similar to that which we have previously reported (16). Body weight did not differ between β-caPKA mice and controls (Supplementary Fig. 1). Insulin sensitivity was similar under all these treatment conditions (Supplementary Fig. 2*A*–*C*), and hepatic glucose production was similar under both basal conditions and in response to a hyperinsulinemic clamp (Supplementary Fig. 2*D*), indicating that these improvements in glucose control are due to improved insulin secretion and not attributable to differences in insulin sensitivity.

Up to 80% of glucose disposal occurs at skeletal muscle (30), and the AIR is an important determinant of the rate of insulin access to the muscle interstitium (31). To determine whether the improved profile of insulin release in β-caPKA mice resulted in enhanced insulin action, fasted mice were killed either prior to a glucose challenge (time = 0) or 5, 10, and 15 min thereafter and quadriceps muscle harvested. Akt phosphorylation/activation in the quadriceps muscles of β-caPKA mice was both stronger and more rapid in β-caPKA mice than in controls (Fig. 3*A*). Consistent with this, 15 min after a radiolabeled glucose challenge, the uptake of [$^{14}C$]-2-deoxyglucose-labeled glucose into gastrocnemius and cardiac muscle in β-caPKA mice was significantly enhanced (Fig. 3*B* and *C*), with quadriceps muscle showing a similar trend (Fig. 3*D*). In mice subjected to a hyperinsulinemic clamp, glucose disposal was similar between β-caPKA mice and littermate controls at both basal conditions and during the last 15 min of the 90-min clamp (Supplementary Fig. 3). Thus, in response to a glucose bolus, the enhancement of the AIR improved glucose uptake into skeletal muscle, but this is not reflective of an underlying difference in glucose uptake rates under the chronically elevated glucose conditions of a hyperglycemic clamp. We conclude that the improved glucose control in response to an increase in PKA activity in the β-cells primarily results from PKA augmentation of the AIR, leading to enhanced insulin action in the periphery.

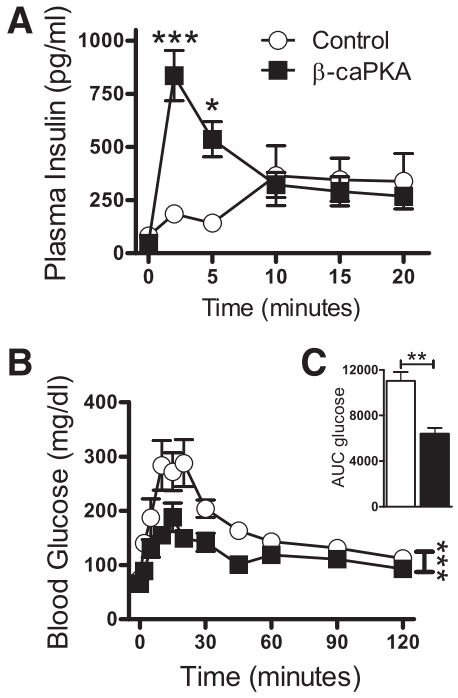


**Figure 1**—Activation of PKA at 13 weeks of age enhances the AIR. Insulin secretion (*A*), glucose tolerance (*B*), and glucose exposure (*C*) in β-caPKA (filled squares) and control mice (open circles) in response to intraperitoneal glucose at 13 weeks of age following tamoxifen administration at 10 weeks of age. Glucose and insulin time courses compared by two-way ANOVA, with Bonferroni posttest analyses. Glucose exposure (*C*) was calculated by the area under the curve of the glucose curves in *B* and compared by Student *t* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. $n$ = 3–5 mice/group. AUC, area under the curve.

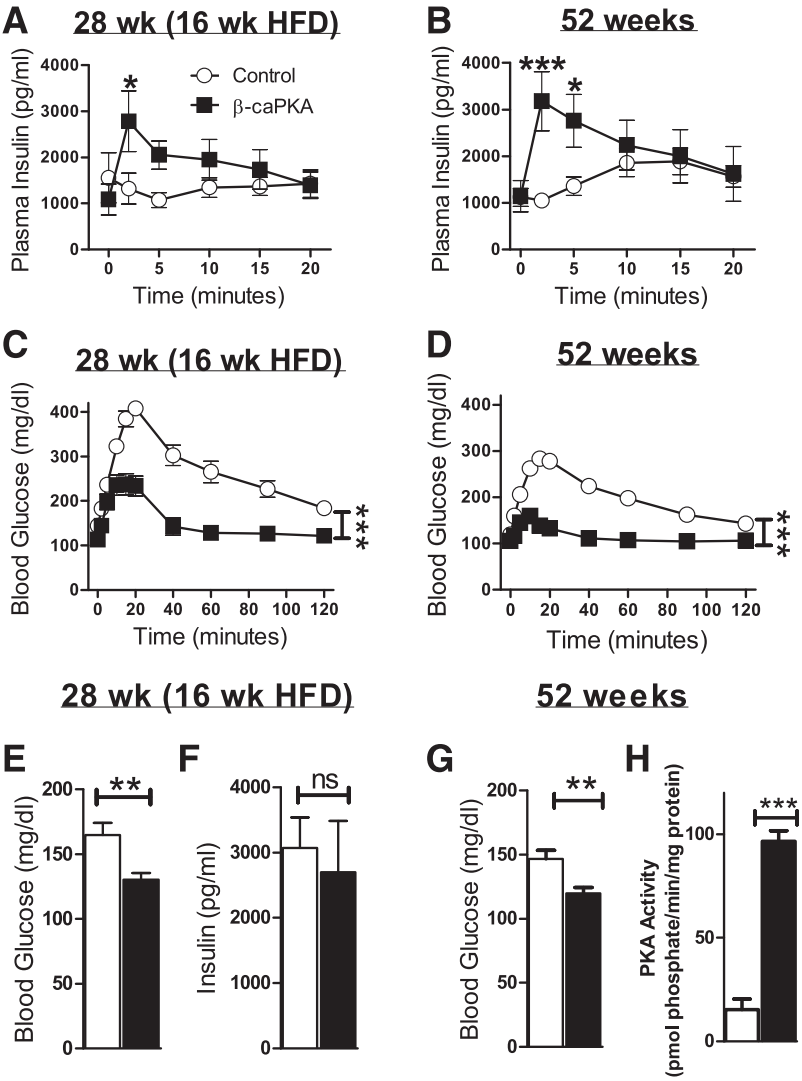


**Figure 2—** PKA activity provides sustained enhancement of the AIR and improved glucose control. Insulin secretion (*A* and *B*) and glucose tolerance (*C* and *D*) in β-caPKA (filled squares) and control mice (open circles) following intraperitoneal glucose at 28 weeks of age following 16 weeks on an HFD (*A* and *C*) and at 52 weeks of age (*B* and *D*). Ad libitum-fed blood glucose and insulin in 28-week-old mice (16 weeks HFD; *E* and *F*) and fed blood glucose in 52-week-old mice (*G*). Enhanced PKA activity in islets isolated from mice at 52 weeks of age, measured in the absence of added cAMP (*H*). *A–D*: Compared by two-way ANOVA, with Bonferroni posttest analyses. *E–H*: Compared by Student *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *A–G*: $n \geq 11$ mice/group. *H*: $n = 5$ mice/group. ns, not significant; wk, weeks.

### PKA Activity Protects From STZ-Induced β-Cell Ablation

Incretin hormones are considered protective for β-cell mass, in part through well-established anti-apoptotic effects. Incretin hormones have also been shown to promote β-cell proliferation and β-cell mass expansion, which has been associated with a rise in β-cell cAMP (17,32,33). To determine whether the activation of PKA enhances β-cell survival, mice that had received tamoxifen at 10 weeks of age were administered STZ at 6 months of age (Fig. 4). Six-month-old mice were used, as it has been reported that proliferative responses to β-cell ablation are minimal in mice of this age (34). In control mice, daily ad-libitum-fed glucose levels increased following STZ (Fig. 4*A*), and the mice became glucose intolerant (Fig. 4*B*). However, β-caPKA mice were protected from both these impairments of glucose control (Fig. 4*A* and *B*). In β-caPKA mice and control mice that did not receive STZ, pancreatic β-cell area was similar, indicating that PKA activity does not cause β-cell hyperplasia (Fig. 4*C*) (0.97 ± 0.66 vs. 0.81 ± 0.55, respectively; $n \geq 3$ mice/group). In control mice administered STZ, β-cell area decreased significantly, whereas in β-caPKA mice, the relative β-cell area was not significantly affected following STZ administration (Fig. 4*C*) (0.22 ± 0.15 vs. 0.64 ± 0.29, respectively; $n \geq 8$ mice/group). These data demonstrate that PKA activity is protective for β-cells challenged with STZ.

To determine whether PKA activity altered the capacity of the pancreas to release insulin, total pancreatic insulin content was measured in mice 13 weeks old, in mice 28 weeks old with 16 weeks of HFD, and mice 52 weeks old. Total pancreatic insulin content was measured so as to gauge the capacity of the pancreas to release insulin by encompassing effects upon insulin content per β-cell and the total number of β-cells in

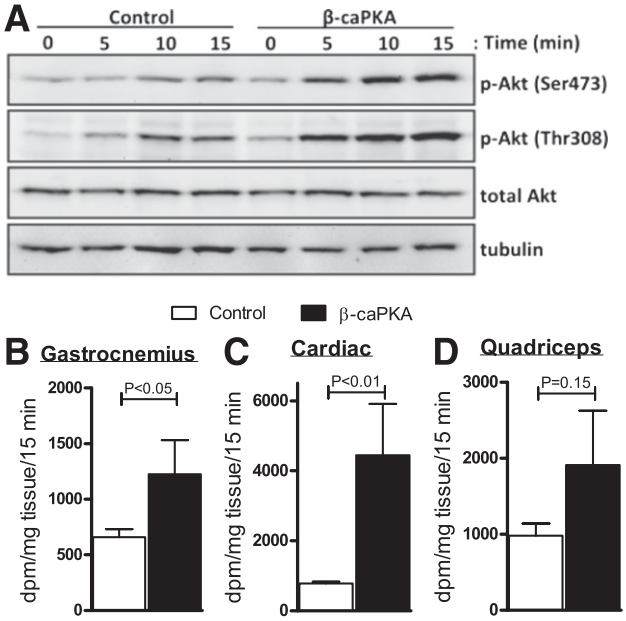


**Figure 3**—Enhanced AIR improved muscle insulin action. *A*: Quadriceps muscle was harvested either prior to a 3 g/kg body weight glucose bolus or 5, 10, or 15 min thereafter in 24-h-fasted β-caPKA and control mice. Lysates prepared from these muscles were immunoblotted for phospho-Akt at Serine 473 and threonine 308, total Akt, and tubulin. Fifteen minutes after a 2-deoxy-D-[1-$^{14}$C]glucose radiolabeled glucose challenge, β-caPKA mice (filled bars) and littermate controls (open bars) were killed and gastrocnemius (*B*), cardiac (*C*), and quadriceps muscle (*D*) harvested. Uptake was quantified as the amount of radioactivity present in muscle tissue (dpm) relative to the muscle mass (mg) over the 15-min labeling period. *B–D*: Compared by Student *t* test; $n$ = 6–9 mice/group. p-Akt, phospho-Akt; Ser473, Serine 473; Thr308, threonine 308.

the entire pancreas. In control mice, pancreatic insulin content increased in association with the HFD and aging (Fig. 4*D*). However, under each of these conditions, there was no significant difference in pancreatic insulin content between control mice and β-caPKA mice (Fig. 4*D*). To determine whether PKA activity promoted an increase in pancreatic β-cell area, pancreata of 52-week-old mice were stained for insulin and the insulin positive area expressed relative to the total pancreas area (Fig. 4*E*). This revealed no significant difference in pancreatic β-cell area between β-caPKA mice and controls (1.1 ± 0.25 vs. 2.0 ± 6.4, respectively; $P$ = 0.2; $n$ = 6 mice/group). The data presented here show that PKA activity is protective for β-cells challenged with STZ, but PKA activity does not result in an increase in the pancreatic β-cell area or the pancreatic insulin content and hence secretory capacity of the pancreas. These data are consistent with a model whereby PKA activity preserves β-cells and improves glucose control solely by enhancing the functional capacity of the β-cells to secrete insulin.

### Enhanced AIR Reverses Preexisting Glucose Intolerance

The above data show that the β-caPKA mice are a model of improved β-cell secretory function, with protective effects upon β-cells but no expansion of β-cell number. To test whether therapeutically enhancing β-cell secretory function can restore impaired glucose control, β-caPKA and control mice were placed on an HFD for 18 weeks, without prior tamoxifen administration, to preestablish insulin resistance and impaired glucose control (Fig. 5). Insulin sensitivity was reduced similarly by aging and high-fat feeding in β-caPKA mice and littermate controls from weeks 9 to 34 (Fig. 5*A*). Consistent with this, glucose tolerance measured at 9 weeks of age (prior to the commencement of the HFD), at 28 weeks of age (after 18 weeks of HFD but prior to tamoxifen administration at 29 weeks of age), and at 34 weeks of age (5 weeks after tamoxifen-mediated induction of PKA activity) declined in control mice (Fig. 5*C*). Following tamoxifen-mediated induction of β-cell PKA activity, the profile of insulin secretion was enhanced in β-caPKA mice with an augmented release peaking at 2 min (Fig. 5*D*), while insulin secretion in littermate controls remained unchanged (Fig. 5*B*). In β-caPKA mice, this resulted in a complete restoration of glucose tolerance to levels similar to those seen at 9 weeks of age, prior to both the HFD and 25 weeks of aging (Fig. 5*E*). In contrast, glucose tolerance in control mice continued to

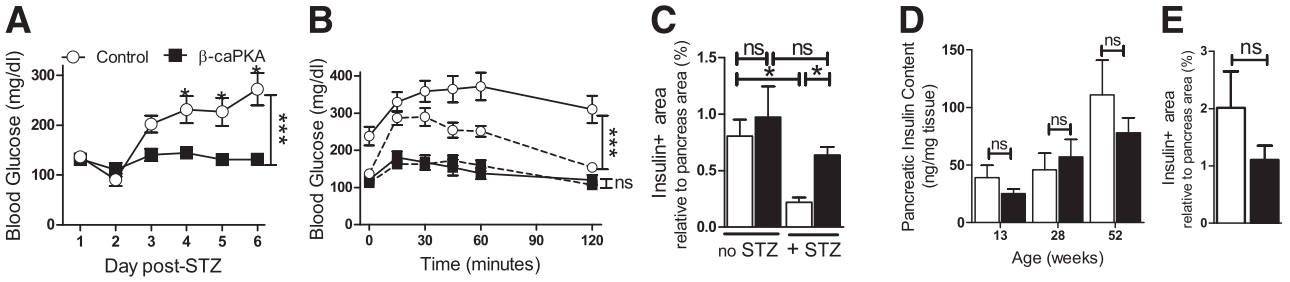


**Figure 4**—PKA activity protects β-cells against STZ but is not associated with β-cell hyperplasia. β-caPKA mice (filled squares/bars) and control mice (open circles/bars) were administered tamoxifen at 10 weeks and aged to 6 months before receiving STZ to challenge β-cell survival. *A*: Daily glucose levels in ad-libitum-fed mice from the day of STZ administration (day 1) until sacrifice (day 6). *B*: Intraperitoneal glucose tolerance measured prior to STZ on day 1 (dotted lines) and again on day 6 (solid lines). *C*: Pancreata harvested from mice ($n$ = 3–8 mice/group) at sacrifice were stained for insulin to calculate the percentage of β-cell area relative to total pancreas area. *D*: Insulin content in extracts prepared from pancreata of β-caPKA (filled bars) and control (open bars) mice killed at 13, 28, or 52 weeks of age ($n$ = 6–18 mice/group). *E*: β-Cell area relative to pancreas area was measured for β-caPKA mice and littermate controls at 52 weeks of age ($n$ = 6). *A* and *B*: Analyzed by two-way ANOVA with Bonferroni post hoc analyses ($n \geq 12$ mice/group in two replicate experiments). *C* and *D*: Analyzed by one-way ANOVA. *E*: Analyzed by Student *t* test. *$P < 0.05$; ***$P < 0.001$. ns, not significant.

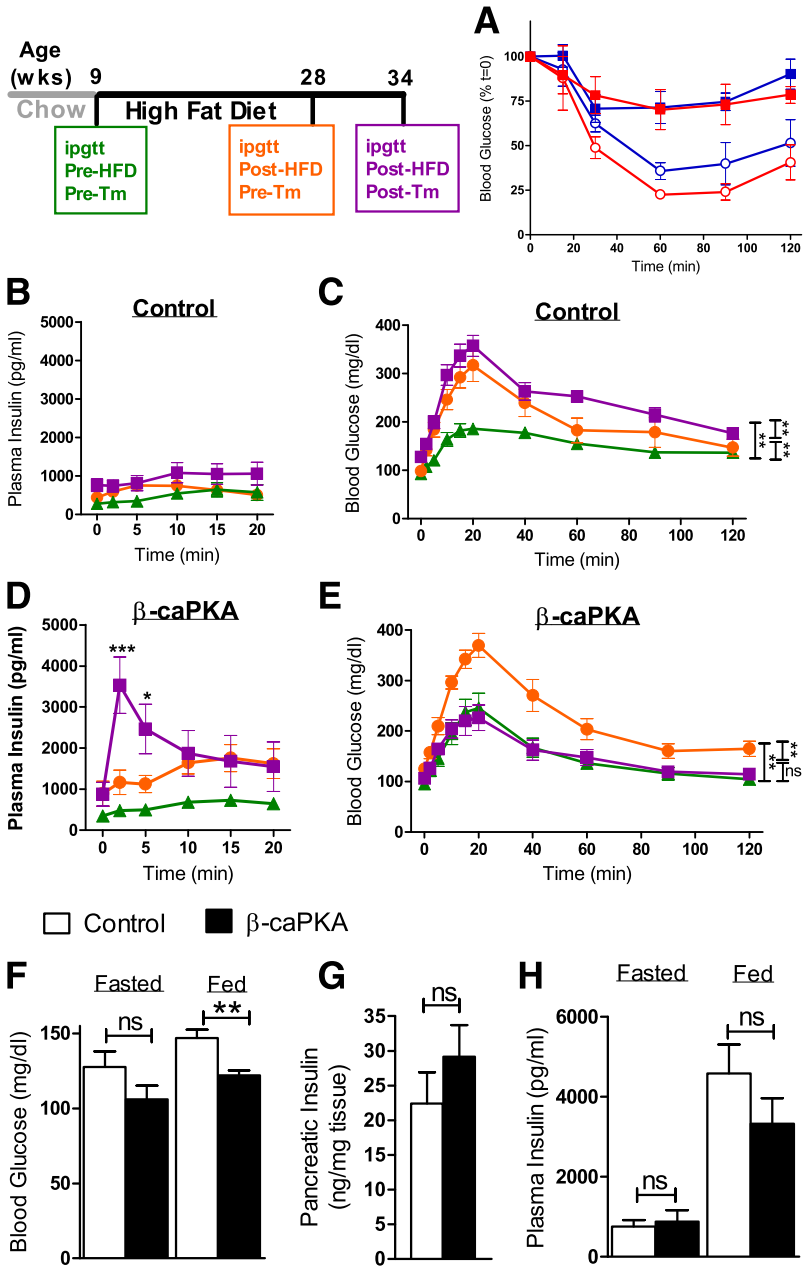


**Figure 5**—Enhanced AIR reverses diet-induced glucose intolerance. β-caPKA mice and littermate controls were maintained on an HFD from 10 weeks of age to the end of the study at 34 weeks of age. Tamoxifen was administered to all mice at 29 weeks of age. IPGTTs were performed at 9, 28, and 34 weeks of age. *A*: Insulin tolerance tests at 9 weeks (open circles) and 34 weeks (filled squares) in β-caPKA (red) and control mice (blue). Plasma insulin (*B* and *D*) and blood glucose (*C* and *E*) from IPGTTs performed at 9 weeks (green triangles), 28 weeks (orange circles), and 34 weeks (purple squares) in β-caPKA (*D* and *E*) and control (*B* and *C*) mice ($n$ = 10–12 mice/group analyzed in two replicate experiments). *F*: Blood glucose in 16-h-fasted and ad-libitum-fed β-caPKA (filled) and control (open) mice at 34 weeks of age ($n$ = 12 mice/ group). *G*: Pancreatic insulin content in 34-week-old β-caPKA (filled bar) and control (open bar) mice ($n$ = 3–6 pancreata/group). *H*: Ad-libitum-fed and 16-h-fasted plasma insulin levels measured in β-caPKA mice (filled bars) and littermate controls (open bars). *A–E*: Analyzed by two-way ANOVA with Bonferroni post hoc analyses. *F–H*: Student $t$ test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ns, not significant; Tm, tamoxifen; wks, weeks.

decline (Fig. 5*C*). The improved glucose control in β-caPKA mice was associated with better ad-libitum-fed glucose levels (Fig. 5*F*). Overnight fasting revealed no difference in glucose levels, consistent with a low risk for hypoglycemia (Fig. 5*F*). Pancreatic insulin content and plasma insulin under fed and fasted conditions did not differ between β-caPKA mice and controls (Fig. 5*G* and *H*), indicating that the improved glucose control in β-caPKA mice is attributable to enhanced β-cell secretory function rather than β-cell hyperplasia or hyperinsulinemia.

To determine whether enhanced β-cell function can also compensate for a reduction in the number of insulin-secreting β-cells, β-caPKA and control mice were treated with STZ prior to tamoxifen administration (Fig. 6). STZ-treated mice with

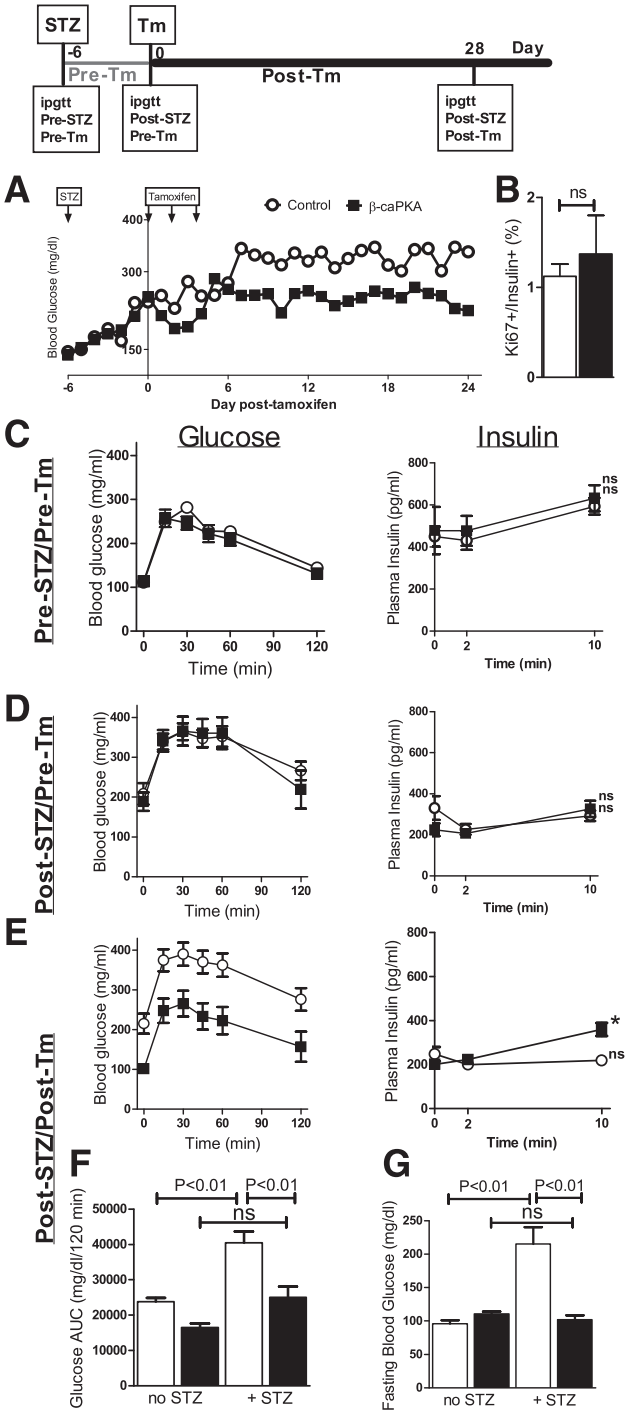


**Figure 6**—Enhanced AIR compensates for depleted β-cell mass. β-caPKA and control mice were aged to 6 months before receiving STZ to deplete β-cell mass. Upon the development of hyperglycemia, tamoxifen was administered (day 0). *A*: Daily blood glucose levels in ad-libitum-fed mice were measured from the administration of STZ (day −6 relative to the start of tamoxifen administration) to sacrifice (day 29 post-tamoxifen; $n$ = 7–8 mice/group in two replicate experiments). *B*: Pancreata from STZ-treated mice ($n$ = 5 mice/group) were analyzed for regenerative β-cell proliferation by co-staining for Ki67 and insulin. Quantification is the number of Ki67$^+$/insulin$^+$ cells relative to the total number of insulin+ cells. Glucose tolerance and circulating insulin were measured at the following times: (*C*) prior to STZ and tamoxifen; (*D*) after STZ but before tamoxifen; and (*E–G*) after the administration of both STZ and tamoxifen in β-caPKA mice (filled squares) and littermate controls (open circles). Area under the curve of the glucose tolerance curves (*F*) and the overnight fasting plasma glucose values (*G*) of STZ-treated mice following tamoxifen administration were compared with values from non-STZ-treated mice. Open bars represent controls. Filled bars represent β-caPKA mice. Comparisons in glucose plots (*A* and *C–E*) are by two-way ANOVA. Comparisons shown in insulin graphs (*C–E*) are $t$ = 10 min vs. $t$ = 0 values by one-way ANOVA. *$P < 0.05$. $n$ = 3–18 mice/group in ≥2 replicate experiments. AUC, area under the curve; ns, not significant; Tm, tamoxifen.

blood glucose levels between 200 and 370 mg/dL were selected for inclusion in this study. Tamoxifen was administered to STZ-treated and untreated mice to induce PKA activity. Daily glucose in ad-libitum-fed mice was significantly lower in STZ-treated β-caPKA mice than STZ-treated controls following tamoxifen administration (Fig. 6*A*) ($P < 0.0001$ by two-way ANOVA; $n \geq 7$ mice/group). In pancreatic sections from these mice, there was no evidence for regenerative proliferation in the β-caPKA mice versus the controls (Fig. 6*B*) (1.37 ± 0.96 vs. 1.12 ± 0.30 Ki67-positive β-cells, respectively; $n$ = 5 mice/group), consistent with the improvement in glucose levels being due to enhanced β-cell secretory function. To determine whether glucose tolerance was also improved, IPGTTs were performed at three time-points: prior to STZ (Fig. 6*C*), after STZ administration but before tamoxifen administration (Fig. 6*D*), and after tamoxifen administration (Fig. 6*E*). Prior to STZ treatment, β-caPKA mice did not differ in glucose tolerance from littermate controls (Fig. 6*C*). Following STZ treatment, both β-caPKA mice and control mice had similarly impaired glucose tolerance relative to pre-STZ glucose tolerance (Fig. 6*D* vs. Fig. 6*C*). Tamoxifen-mediated induction of PKA activity in STZ-treated β-caPKA mice improved glucose tolerance (Fig. 6*E*) such that it was not significantly different to β-caPKA mice that did not receive STZ (Fig. 6*F*). Similarly, overnight fasting blood glucose was restored to levels similar to those of non-STZ-treated mice (Fig. 6*G*). Insulin levels were significantly diminished in both β-caPKA mice and controls following STZ treatment (Fig. 6*D*). However, following tamoxifen-mediated induction of PKA activity, glucose stimulation significantly potentiated circulating insulin levels in β-caPKA mice but not controls (Fig. 6*E*). These data demonstrate that therapeutic enhancement of insulin release via PKA activation can restore glucose control under conditions of preexisting glucose intolerance.

### PKA Activation Does Not Lead to Hyperinsulinemia or Hypoglycemia

It is necessary that therapies targeting insulin release do not impair the tight control of insulin secretion and avoid therapeutic complications such as hyperinsulinemia and hypoglycemia. To determine whether the enhanced insulin secretion in response to PKA activation leads to hyperinsulinemia, β-caPKA mice and littermate controls were aged to 52 weeks, with circulating insulin measured at regular intervals. In both fasting mice (Fig. 7*A*) and

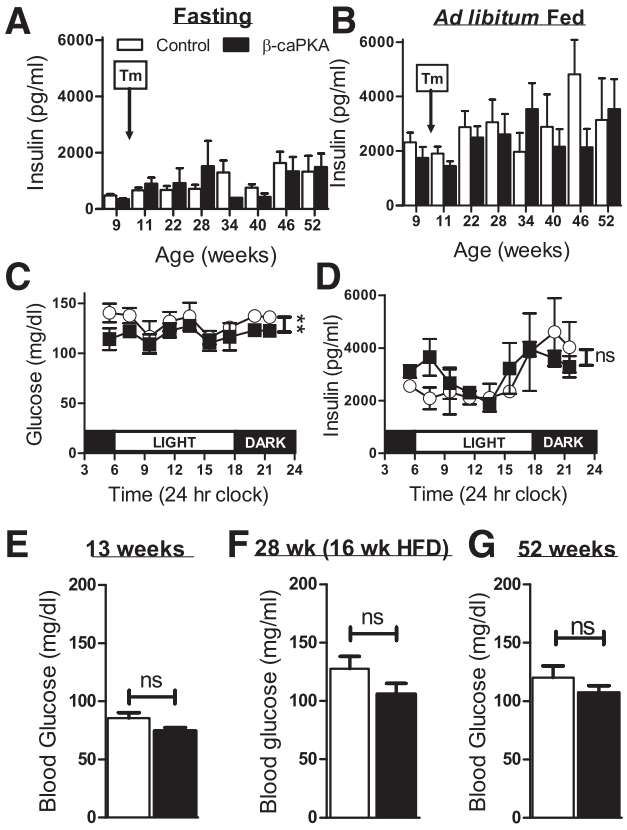


**Figure 7**—β-caPKA mice do not exhibit hyperinsulinemia or hypoglycemia. β-caPKA mice (filled bars) and littermate control mice (open bars) were administered tamoxifen at 10 weeks and then aged to 52 weeks with regular measurements of fasting (*A*) and ad-libitum-fed (*B*) plasma insulin levels (two-way ANOVA; $P > 0.05$ for both; $n \geq 6$). β-caPKA mice (filled squares) and littermate controls (open circles) administered tamoxifen at 10 weeks and aged to 4 months were allowed to feed ad libitum throughout a 24-h period, with blood samples taken for measurements of blood glucose (*C*) and plasma insulin (*D*) from 5:30 A.M. to 11:30 P.M. on a 12-h light/12-h dark cycle with the light cycle commencing at 6:00 A.M. Sixteen-hour fasted blood glucose levels were measured at 13 weeks of age (*E*), 28 weeks of age after 16 weeks of an HFD (*F*), and 52 weeks of age (*G*) in β-caPKA mice (filled bars) and littermate controls (open bars) to determine whether β-caPKA mice had an increased risk for hypoglycemia. *A–D*: Compared by two-way ANOVA. *A* and *B* were not significantly different; $n \geq 3$ mice/group. *E–G*: Compared by Student *t* test; $n = 5–15$ mice/group. $^{**}P < 0.01$. ns, not significant. Tm, tamoxifen; wk, weeks.

ad-libitum-fed mice (Fig. 7*B*), insulin levels did not differ. To further explore this issue, β-caPKA and control mice were monitored throughout a single day for ad libitum plasma insulin and blood glucose levels (Fig. 7*C* and *D*). As expected, glucose control was significantly improved in β-caPKA mice relative to controls (Fig. 7*C*), but this was not associated with differences in circulating insulin (Fig. 7*D*). To determine whether the enhanced insulin release in β-caPKA mice raises the likelihood of hypoglycemia, mice were fasted overnight, and blood glucose was measured. Fasting blood glucose levels did not differ between β-caPKA mice and controls at three ages: 13 weeks of age (Fig. 7*E*); 28 weeks of age with 16 weeks of an HFD (Fig. 7*F*); and 52 weeks of age (Fig. 7*G*). These data show that chronic hyperinsulinemia does not underlie the improved glucose control in β-caPKA mice, nor does the enhancement of insulin secretion by PKA activation lead to unregulated insulin release resulting in hyperinsulinemia or hypoglycemia.

## DISCUSSION

Insulin is secreted from the β-cells with a biphasic profile in which the acute burst of release, which lasts 5–10 min, is followed by a sustained phase that continues while glucose remains elevated. Acute insulin is a major determinant of the efficacy of glucose clearance (11) through its ability to improve insulin action to shut down hepatic glucose output and to accelerate insulin translocation from the circulation into the muscle and adipose tissue (30,31,35–37). Insulin access to myocytes and adipocytes is enhanced by the potentiation of the AIR through improved transendothelial transportation of insulin from the circulation and via increased blood flow in muscle and adipose tissue (30,31,37). The association of impaired AIR with the early stages of T2DM and its importance in determining the rate of glucose clearance make its improvement an attractive target for therapies to restore β-cell function (38,39). Our characterization of the β-caPKA mice both here and previously (16) show that these mice are a model of inducible augmentation of the AIR through PKA activation. The data we present here support the model whereby the enhancement of the AIR improves insulin action in skeletal muscle and accelerates glucose uptake. Here we show that shifting the profile of insulin release to enhance acute phase release reverses preexisting glucose intolerance arising from high-fat feeding. In addition, the induction of enhanced insulin secretion is able to restore glucose control in mice in which glucose control has become impaired following β-cell mass depletion by STZ. These data indicate the potential that exists within enhancement of β-cell secretory function to overcome impaired glucose control, without the need to expand β-cell number. Earlier studies have shown that β-cell hyperplasia can prevent (40,41) and reverse (42) glucose intolerance. Here we show an alternative approach, whereby alteration of the profile of insulin release is sufficient to regain glucose control. The β-caPKA mice do not provide evidence for PKA activity in the expansion of β-cell number. Measurements of β-cell area relative to pancreas area, total pancreatic insulin content, and Ki67 staining for regenerative proliferation following STZ-mediated depletion of β-cell mass provide no indication that the increase in PKA activity is associated with β-cell expansion or proliferation. These data do not preclude a role for PKA signaling in β-cell proliferation, but they do demonstrate that the activation of PKA is not sufficient to drive an expansion of β-cell number. Indeed it has been proposed that the elevated glucose associated with an insufficient β-cell mass may be a contributing factor to β-cell proliferation. Therefore the tighter glucose control in the β-caPKA mice may lessen any proliferative actions of

PKA. Interestingly, it has been reported that the tighter glucose control arising from incretin action to enhance insulin release may lessen the expansion of β-cell mass (43). Importantly, the PKA-mediated restoration of glucose control, via enhancement of acute insulin release, was achieved without hyperinsulinemia or risk for hypoglycemia, two complications that can be associated with excessive insulin release, and indicates that PKA-mediated enhancement of β-cell function does not lead to a loss of the tight control over insulin release that is evident with models of β-cell hyperplasia. Moreover, the PKA-mediated enhancement of insulin secretion was sustainable, showing no evidence for β-cell exhaustion out to 52 weeks of age. This may in part be attributable to the protective effects of PKA activity that we observe for β-cells challenged with STZ. However, this may also be due to the more efficient profile of insulin release and more rapid restoration of euglycemia, placing less of the stress upon β-cells that occurs during prolonged secretion (44). The insulin secretion potentiated by PKA activity does not result in chronic hyperinsulinemia, but rather it delivers acute enhancement of insulin secretion to rapidly lower glucose levels. Overall, these data, showing normal fasting glucose and circulating insulin levels with the sustainability of the effect, demonstrate the benefits to diabetes therapy of altering the profile of insulin secretion, rather than causing bulk increase in insulin release. New areas of research are advancing the study of GPCRs for therapeutic uses. Novel receptors and ligands, better understanding of their biology, and the developing areas of bias ligands and small molecule allosteric regulators of GPCR signaling will create opportunities to target specific pathways downstream of GPCRs (26,45,46). To select specific beneficial downstream outcomes requires an understanding of the risks and benefits of downstream signaling systems. Here we demonstrate that cAMP signaling via PKA in β-cells provides beneficial effects to insulin secretion and the protection of β-cell mass with a low risk for adverse effects.

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**Author Contributions.** K.A.K., L.M.D., and B.W. conceived and conducted this study, interpreted these data, and prepared the manuscript. J.H.E. and C.M.D.O. assisted with technical aspects of experiments. B.T.L. interpreted these data and prepared the manuscript. B.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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